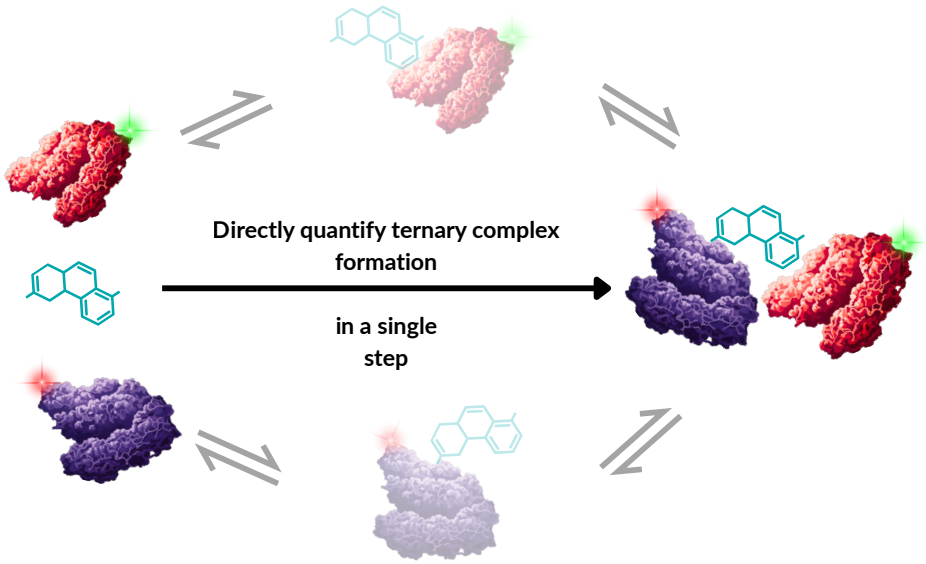
Directly quantify ternary complex formation in solution

Application note

In this application note, we demonstrate how to directly characterise ternary complexes in solution, using fluorescence cross-correlation spectroscopy (FCCS) on the EI-FLEX Pro system. FCCS is used to generate hook plots to establish the optimal concentration of an unlabelled bridging molecule to produce the desired stoichiometry and avoid unwanted dimers. The impact of salt concentration on hybridisation kinetics is also investigated. All data was generated in-house at Exciting Instruments.

FCCS uses two different-coloured lasers to determine whether two biomolecules (such as a drug and its target) are moving through the solution as a single unit. It is ideal for studying ternary complexes, such as PROTACs, molecular glues, and bispecific antibodies. In this application note, a model DNA oligo system was used for the purposes of demonstrating the underlying measurement principles.



Overview of this application note:

- FCCS provides a direct quantification of ternary complexes in solution, as cross-correlation is only detected when all three components are present in the same complex
- Hook plots produced by titration of the unlabelled oligo identify the optimal concentration for ternary complex formation
- Kinetics of ternary complex formation can be directly calculated using FCCS

Generate hook plots for ternary complexes in solution

One of the key experimental outputs when characterising ternary complexes is identifying the optimal concentration of the bridging biomolecule that maximises the desired stoichiometry. Dose-response curves for ternary complexes are not linear, resulting in the formation of unwanted binary complexes at increasing concentrations of the bridging molecule. This is known as the 'hook-effect' and is often challenging to characterise, as the concentrations at which positive hits can be identified tend to be narrow, leading to false negatives during screening.

FCCS is an ideal technique for producing hook plots; cross-correlation provides a direct readout of ternary complex formation across a titration of the bridging molecule at pM-nM concentrations. Using our model oligo system, we performed a titration of our bridging molecule (oligo 2) using equal concentrations of oligos 1 and 3. Two hook plots were generated, one at 500 pM of labelled oligos and the other at 20 nM of labelled oligos (Figure 1). These values showed good agreement with simulated hook plots derived from the model for three-body binding equilibria as published by Douglass et al.[1]

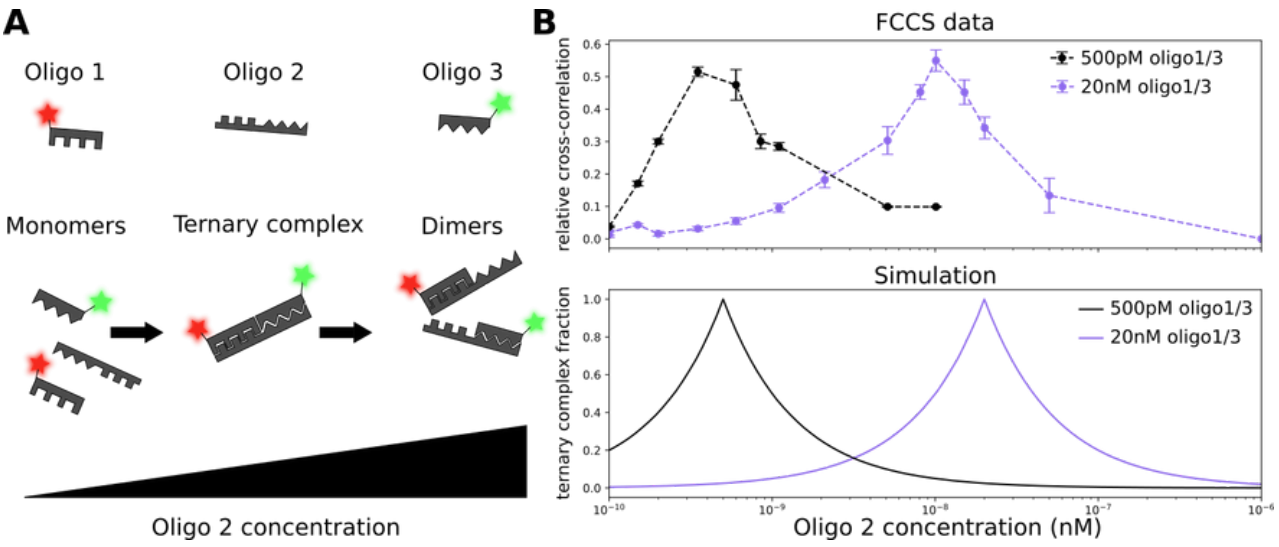


Figure 1 – Hook plots generated using FCCS analysis of ternary complexes
(A) Schematic of the dose-response to the ternary-complex inducing oligo 2. With increasing concentration of oligo 2, a ternary complex is formed; at saturating concentrations, only dimers of oligo 1/3 and oligo 2 are formed.
(B) Top: Dose-response curve at 500pM (black) and 20nM (purple) oligo 1/3.
Bottom: Calculated dose-response curves using the model developed by Douglass et al.[1]

Directly measure kinetics of ternary complex formation

Another important characteristic of ternary complexes is the kinetics of their formation. In other methods, such as SPR, ternary complex kinetics are characterised by measuring the individual binary rates that make up the total reaction. Using FCCS, we have direct access to the ternary complex formation kinetics in solution. Figure 2 shows the formation of our DNA-based ternary complex for three different concentrations of NaCl, whereby the molecular association rates increase with salt concentration.

To quantify the change in rate, we fit the data to an analytical rate equation of ternary complex formation with the following simplifying assumptions:

- The initial concentrations of oligos 1 and 3 are equal
- The reaction step is irreversible since off-rates for long DNA molecules are known to be extremely slow
- All intermediate reaction steps have identical rates

This last assumption neglects any sequence dependency that the reaction rates might display. However, when we derived a model under these circumstances, it fit our data sufficiently well, and the extracted rate increased with NaCl concentration as expected.

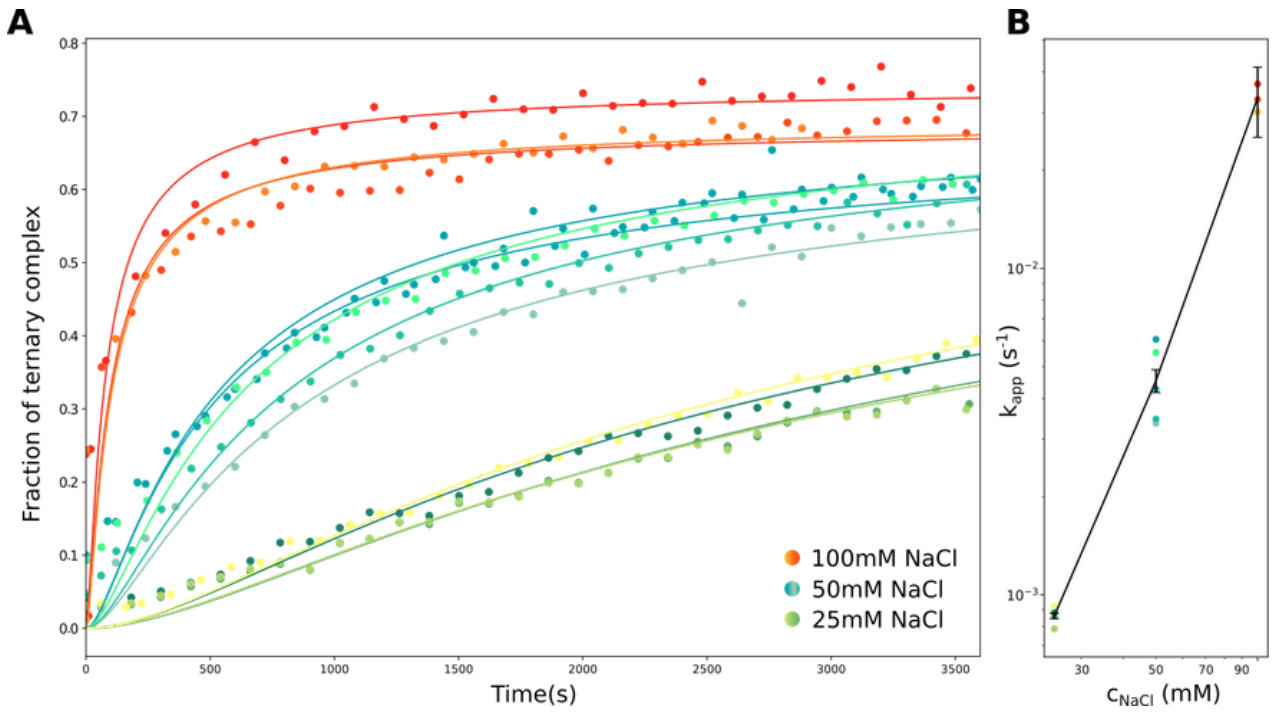


Figure 2 – Effect of NaCl concentration on ternary complex formation

(A) Time course experiments of ternary-complex formation at 25, 50 and 100 mM NaCl. The dots represent cross-correlation amplitudes normalised to a positive control, with lines fitted to an analytical rate equation, assuming all kinetic rates and initial conditions are equal, and irreversible binding occurs. (B) Apparent reaction rates extracted from (A) (coloured dots). The black points/bars represent the mean and standard error of the mean, respectively.

FCCS provides a direct readout of ternary complex formation

FCCS provides a direct readout of ternary complex formation by measuring cross-correlation (Figure 3). In this example, oligo 1 was labelled with a green fluorophore and oligo 3 was labelled with a red fluorophore; they only produce a cross-correlation signal when they are diffusing together as a ternary complex bound to unlabelled oligo 2.

The change in cross-correlation was measured over time and in response to increasing concentrations of oligo 2, resulting in greater cross-correlation over a shorter time period. However, the highest cross-correlation amplitude does not correlate with the concentration of oligo 2; under saturating conditions, binary complexes dominate and prevent the formation of ternary complexes.

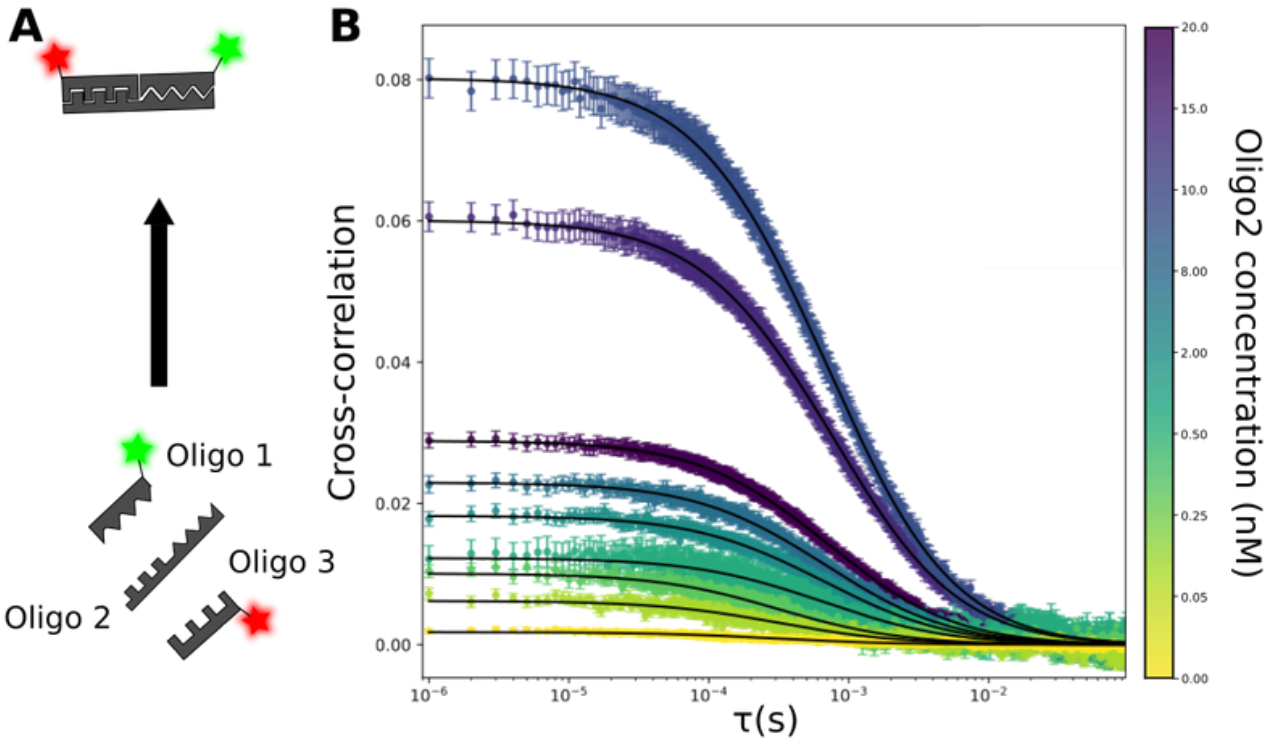


Figure 3 – Cross-correlation provides a direct readout of ternary complex formation
(A) Schematic of ternary complex formation of oligo 1 (green label), oligo 2 (no label) and oligo 3 (red label). (B) Cross-correlation measured on the EI-FLEX with 20nM oligos 1 and 3 and varying concentrations of oligo 2 (coloured datapoints). The data was fitted with a diffusion model accounting for possible triplet states (black lines). All measurements were acquired in TE buffer supplemented with 150mM NaCl and 1% glycerol.

Practical considerations for quantifying ternary complex formation

- Label two components of the ternary complex with different colours to allow analysis via fluorescence cross-correlation spectroscopy (FCCS)
  - Detection of co-diffusion permits the direct quantification of ternary complex formation and hook plot calculation without the need to establish binary kinetics first
- Samples are analysed in solution, avoiding surface-induced artefacts, and capturing ternary complex fractions as low as ~1-3 % across pM-nM concentration regimes
- Experiments are performed in 96- or 384- well plates and can be completed within hours, requiring around 40-100 µL of sample per well with acquisition times of ~60 seconds per sample

The EI-FLEX Pro System

The EI-FLEX Pro provides fully integrated, high-throughput workflows, with automated sample positioning, focus maintenance and acquisition of 96- and 384-well plate formats. Guided sample acquisition and analysis are performed using dedicated software and bespoke workflows.

The EI-FLEX Pro single-molecule spectrometer

Summary

FCCS is an ideal technique for characterising ternary complexes, providing a direct, quantitative readout of ternary complex formation and enabling the generation of hook plots to determine optimal therapeutic concentrations. The rapid acquisition of samples in solution enables scalable data collection and iterative testing under physiologically relevant conditions, without the need for immobilisation or complex microfluidics.

For a deeper dive on the techniques used in this application note, we recommend exploring our Resource Library. Discover a range of applications for FCCS and the EI-FLEX system on our website.

References

1. Douglass, E. F. Jr., Miller, C. J., Sparer, G., Shapiro, H. & Spiegel, D. A. A Comprehensive Mathematical Model for Three-Body Binding Equilibria. J. Am. Chem. Soc. 135, 6092–6099 (2013).

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